



No. 03-021871- -00006-0000

Fecha: 2007-10-02 16:19:59 Dep. 2020 NUECREACIONE
Tra. 11 PATENTEIYII Eve: 379 FASENACIONALII
Act. 444 RESPUESTAREQUE Folios: 23

Señores

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIVISIÓN DE NUEVAS CREACIONES

E. _____ S. _____ D. _____

Ref: Expediente No. 03.021.871

Solicitud de patente de invención titulada:

“VACUNA DE ANTICUERPOS POLISACARIDOS BACTERIANOS”.

Solicitante: SMITHKLINE BEECHAM BIOLOGICALS S.A.

RESPUESTA AL OFICIO No. 7917 NOTIFICADO POR FIJACIÓN EN LISTA No. 235,
DE FECHA 05 DE JULIO DE 2007
(EXAMEN DE FONDO)

J. IAN RAISBECK, mayor de edad y vecino de Bogotá, identificado tal como aparece al pie de mi firma, en mi carácter de en mi carácter de apoderado de la sociedad solicitante, tal como consta en la sustitución de poder adjunta, me permito manifestar lo siguiente ante el auto emanado por ese Despacho, dentro del plazo legal previsto en el artículo 45 de la Decisión 486:

1. En primer lugar y con el ánimo de brindar mayor claridad a la materia reivindicada, me permito presentar un **Capítulo Reivindicatorio Modificado**, que reemplaza en su totalidad al Capítulo Reivindicatorio radicado al momento de presentación de la presente solicitud. Para tal efecto, adjunto un recibo de pago que cubre la tasa de modificación correspondiente.

En el **Capítulo Reivindicatorio Modificado** se realizaron las siguientes modificaciones:

a- Se **ELIMINARON** los términos “por lo menos” y “equivalente” con relación a los componentes antígenos proteicos de las reivindicaciones.

b- La materia de la Reivindicación 1 se **LIMITÓ** a los antígenos PhtA, PhtB, PhtD y PhtE y a polisacáridos de los serotipos 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F y 33F. El soporte para esta modificación se encuentra en la página 7, líneas 9 a 28.

OLARTE RAISBECK

OLARTE RAISBECK FRIERI MOURE & CASTRO LTDA.

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STARIA

27 Bogotá D.C.

c- La Reivindicación 4 se **MODIFICÓ** de manera que ahora requiere necesariamente por lo menos 7 polisacáridos. El soporte para esta modificación se encuentra en página 7, líneas 14 a 18.

d- La Reivindicación 5 se **ELIMINÓ**.

e- La Reivindicación 6 se **RENUMERÓ** como nueva Reivindicación 5 y se **MODIFICÓ** con el fin de incluir los agentes: gel de hidróxido de aluminio o fosfato de aluminio. El soporte para esta modificación se encuentra en la página 18, líneas 4 a 7.

f- Las anteriores Reivindicaciones 7 y 8 se **FUSIONARON** como nueva la Reivindicación 6.

g- Las Reivindicaciones 10, 12-14 y 16 fueron **ELIMINADAS**.

i- Se **MODIFICÓ** la antigua Reivindicación 15 para incluir solamente los antígenos PhtA, PhtB, PhtD y PhtE y se **RENUMERÓ** como nueva Reivindicación 7.

Valga anotar que las modificaciones efectuadas al Capítulo Reivindicatorio cumplen a cabalidad con lo estipulado en el Artículo 34 de la Decisión 486 de la CAN, ya que éstas de ninguna manera implican una ampliación de la protección correspondiente a la divulgación contenida en la solicitud inicial.

2. a- El Examinador argumenta que los términos “al menos” y “equivalente” en las reivindicaciones son imprecisos e indefinidos.

b- Considero superada la anterior objeción, en tanto que los términos “al menos” y “equivalente” fueron eliminados de las reivindicaciones en cuanto a la definición de los antígenos proteicos se refiere. Valga anotar que respecto de los polisacáridos antigénicos, el término “al menos” se mantiene, debido a que las composiciones necesariamente deben contener por lo menos uno de ellos y en el caso de la Reivindicación 7, debe contener por lo menos 7 de ellos.

Es importante recordar que el solicitante de una patente de invención está en su derecho de reclamar protección para la totalidad de un concepto inventivo desarrollado, que no debe restringirse de manera subjetiva solamente a modalidades específicas de la invención, siempre y cuando modalidades adicionales presenten el efecto técnico reclamado. En el presente caso, como lo enseñaré más adelante (ver punto 4.d-), las pruebas experimentales aportadas demuestran que

OLARTER AISBECK
OLARTER AISBECK FRIERI MOURE & CASTRO LTDA.
JURADO DE INVENCIÓN
SECRETARÍA DE ECONOMÍA
D.F.
27 de mayo de 2000

Combinación de diversos números de polisacáridos antígenos proporcionan el efecto sinérgico deseado en las composiciones inmunogénicas desarrolladas.

3. a- El Examinador argumenta que las reivindicaciones reclaman un sinnúmero de composiciones resultantes de la combinación de todos los posibles polisacáridos con todos los posibles antígenos, lo que podría no estar enteramente soportado por la descripción y no conservar un único concepto inventivo. Así mismo, las reivindicaciones que reclaman adicionalmente un coadyuvante (Reivindicaciones 5 a 8) resultarían especulativas ya que el adyuvante podría no estar suficientemente divulgado en la descripción.

b- Llamo respetuosamente la atención del Examinador al hecho que el Capítulo Reivindicatorio Modificado adjunto ahora se limita estrictamente en la Reivindicación 1 a composiciones inmunogénicas que pueden comprender únicamente los antígenos proteicos PhtA, PhtB, PhtD y PhtE y a polisacáridos antigénicos de por lo menos cuatro de los serotipos 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F y 33F, y por lo menos siete de éstos en la Reivindicación 4. Por su parte, las Reivindicaciones 5 y 7 ahora especifican claramente que el adyuvante contemplado en la invención corresponde exclusivamente a gel de hidróxido de aluminio o fosfato de aluminio que puede contener un vehículo seleccionado de una emulsión aceite en agua, liposomas o una sal de aluminio.

Según lo anterior, considero que el alcance de las reivindicaciones se ha reducido significativamente y que el objeto de la presente invención es suficientemente claro, de acuerdo a lo establecido por el Artículo 30 de la Decisión 486.

3. a- El Examinador argumenta que las reivindicaciones 12-14 y 16 recaman materia no patentable por dirigirse a usos y métodos de tratamiento.

b- Considero superada esta objeción en tanto que el Capítulo Reivindicatorio Modificado no contiene reivindicaciones dirigidas a usos o métodos de tratamiento.

4. a- El Examinador considera que los siguientes documentos afectan el nivel inventivo de la presente solicitud:

No.	Documento	Fecha
D1	WO 90/06951	18-06-1990
D2	WO 99/03884	28-01-1999
D3	WO 98/18930	07-05-1998

D1 revela una vacuna que comprende polisacáridos de cápside de *S. pneumoniae* conjugados con una proteína carrier (pneumolisina modificada). Así mismo revela composiciones donde el antígeno proteico no se encuentra modificado.

D2 se refiere a una vacuna que comprende un polipéptido (pneumolisina) conjugado a antígenos polisacáridos provenientes de *S. pneumoniae*.

El Examinador concluye que la diferencia entre el estado de la técnica y la presente solicitud radica en que el carrier específico se selecciona de PhtA, PhtD, PhtB, PhtE, SpsA, LytB, LytC, LytA, Sp125, Sp101, Sp128, Sp130y Sp133. Sin embargo D3 reveló vacunas que contienen antígenos peptídicos provenientes de *S. pneumoniae*, dentro de los que se encuentran el Sp101 y el Sp125. Igualmente, los diferentes antígenos peptídicos de *S. pneumoniae* son bien conocidos en el arte, así como los diversos adyuvantes de las Reivindicaciones 8 y 9.

Así, una persona medianamente versada en la materia conocería a partir del estado del arte el concepto de combinar antígenos polisacáridos de *S. pneumoniae* con antígenos proteicos provenientes de la misma especie para incrementar la inmunogenicidad en vacunas para el tratamiento de neumonía y otitis. Igualmente, el método de la Reivindicación 15, constituye los principios de cualquier proceso de formulación. Por lo tanto, la presente invención carecería de nivel inventivo.

b- i) En primer lugar, me permito adjuntar los resultados de dos experimentos que compararon el efecto de inmunización en ratones con PhtD, inmunización pasiva con anticuerpos anti-polisacáridos, o la combinación de la inmunización con PhtD e inmunización pasiva a polisacáridos.¹ En ambos experimentos, los anticuerpos contra PhtD o polisacáridos, condujeron a una protección leve contra el desafío letal del ataque con una cepa apropiada de *S. pneumoniae*.

Por el contrario, la combinación de anticuerpos contra PhtD y polisacáridos resultó proporcionar una protección completa. En efecto, los resultados mostrados en el Anexo 1 demuestran que la combinación de PhtD y un polisacárido neumocócico, ciertamente proporciona un efecto sinérgico en la respuesta inmune, resultando en una protección completa.

ii) Según lo anterior, la presente invención encontró sorpresivamente que mediante la estimulación simultánea del sistema inmune mediado por actividad celular (por ejemplo, la inmunidad mediada por células T) en adición a la actividad humoral del sistema

¹ Anexo 1

inmune (mediada por células B), genera un efecto sinérgico que es capaz de aumentar la eliminación de neumococo del huésped.²

Así pues, la presente invención enseña que el efecto sinérgico puede ser alcanzado a través de la inmunización con un polisacárido de neumococo y una proteína de neumococo, seleccionados de un grupo particular.

iii) Dado que la familia de proteínas Pht comparte un alto grado de homología, es muy probable que otros miembros de la familia Phtx proporcionen resultados similares cuando se combinan con polisacáridos neumocócicos en una vacuna. La familia Pht se caracteriza por que sus miembros comparten características estructurales entre sus miembros. Como se enseña en la página 10, líneas 4-12 de la presente solicitud, estas proteínas contienen “una secuencia de lipidación, dos dominios separados por una región rica en prolina y muchas triadas de histidina, regiones coiled-coil (doble alfa-hélice), un N-terminal conservado y una C-terminal heterogéneo”. Así las cosas, dado el alto grado de relación entre proteínas Phtx, es de esperarse que vacunas basadas en combinaciones que contienen un polisacárido pneumocócico y una proteína Phtx, proporcionen buenos resultados de protección.

c- Ahora bien, con relación al arte previo citado por el Examinador, es importante subrayar lo siguiente:

i) D1 ciertamente puede considerarse como el arte previo más cercano, puesto que allí se revela un polisacárido de *S. neumoniae* conjugado con pneumolisina alterada, como proteína portadora. D2 igualmente se refiere a vacunas que comprenden un polipéptido (pneumolisina) conjugado con un polisacárido antigénico de *S. neumoniae*. Sin embargo, la diferencia esencial entre lo revelado por D1 y la Reivindicación 1 de la presente solicitud, es la presencia específica de PhtA, PhtB, PhtD y PhtE en la combinación de la vacuna.

El problema técnico objetivo abordado por la presente invención, es la adaptación de la vacuna revelada en D1 para el desarrollo de una vacuna que use tanto la respuesta humoral, como la respuesta celular del sistema inmune, para generar una protección mejorada contra *S. pneumoniae*.

De la lectura de D1, una persona medianamente versada en la materia estaría motivada a usar pneumolisina alterada en conjugación con polisacárido capsular de *S. pneumoniae* en una vacuna. Ahora bien, la pneumolisina alterada es sustancialmente no tóxica, aunque desencadena una respuesta inmune contra la pneumolisina de tipo silvestre (pag. 4. líneas 25-29). Sin embargo, no

² Ver final de la página 3 del Anexo 1.

hay sugerencia alguna en D1 respecto que otra proteína antigénica de *S. pneumoiae* pueda ser aplicable o requerida en la formulación de una vacuna mejorada, así como tampoco acerca de las características que debería tener dicha proteína adicional. Por lo tanto, la vacuna de la Reivindicación 1 no resulta de ninguna manea obvia en vista de la materia revelada por D1, en particular de acuerdo a los excelentes resultados de protección contra infecciones de *S. pneumoniae*, luego de una inmunización como se enseña en el Anexo 1.

ii) Por su parte, D3 se refiere a las proteínas Sp125 y Sp101, que fueron eliminadas del Capítulo Reivindicatorio Modificado adjunto. Sin embargo, la proteína Sp042 corresponde a un miembro de la familia de Pht y también es revelado en D3. De hecho, D3 reveló las secuencias de Sp042 entre un amplio grupo 113 de proteínas de *S. pneumoniae*. Así, D3 igualmente sugiere que una o más de las proteínas reveladas podría ser usada en una vacuna (pag. 36, líneas 7-16).

A pesar de lo anterior, D3 no revela resultados que demuestren la inmunogenicidad o la habilidad de dichas proteínas ara proteger contra *S. penaumoniae*. Una persona medianamente versada en la materia no encontraría indicación alguna en D3 acerca de cuál de las secuencias de polipéptidos allí contenidas generaría una respuesta inmune efectiva contra *S. pneumoiae*. Por lo tanto, una persona medianamente versada en la materia no podría concebir una expectativa razonable para esperar que una de esas proteínas, elegida aleatoriamente constituiría un inmunógeno efectivo.

c. i) En conclusión, la Reivindicación 1 de la presente invención es completamente inventiva respecto de D1, aún en combinación con D3, puesto que no hay indicación alguna en dichas referencias respecto de cuál de las 113 proteínas de *S. pneumoniae* debería utilizarse con el fin de generar una excelente protección contra este patógeno.

ii) La combinación de un polisacátido capsular con una proteína de la familia Pht es capaz de generar una respuesta tanto celular como humoral en el sistema inmune, con el fin de generar una protección casi completa frente a una dosis letal de *S. pneumoiae*. Esta enseñanza no estaba en ningún momento presente o sugerida en D1 o D3, o en la combinación de estas referencias. En conclusión, la presente invención es inventiva frente al arte previo citado por el Examinador.

d- El Anexo 1 demuestra claramente la respuesta sinérgica lograda cuando se combinan polisacáridos neumocócicos y proteínas antigénicas del tipo Pht en una sola vacuna.

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Adicionalmente, me permito adjuntar el Anexo 2 que enseña resultados adicionales ilustrando que el mismo concepto puede aplicarse en el desarrollo de vacunas más complejas que contienen múltiples polisacáridos neumocócicos y múltiples proteínas.

Los ensayos experimentales del Anexo 1 usaron PhtD como proteína libre y los ensayos del Anexo 2 muestran que la PhtD resulta igualmente efectiva cuando se utiliza como una proteína conjugada. Asimismo, los experimentos en el Anexo 1 se realizaron usando un solo polisacárido, sin embargo, las vacunas de la invención también son efectivas cuando se utilizan múltiples polisacáridos. Así, el Ejemplo 4 usa conjugados de 11 polisacáridos y 13 polisacáridos en vacunas que contienen Pht, mientras que los Ejemplos 5, 6 y 8 usan vacunas con 13 polisacáridos.

5. Para terminar, si bien es cierto que las concesiones de patentes en oficinas de otros países no son vinculantes en el sentido que obliguen a la Superintendencia a tomar una decisión en el mismo sentido, sí considero que dichas concesiones sugieren el cumplimiento de los requisitos de patentabilidad de la materia reivindicada, ya que los criterios de evaluación y estudio aplicados para determinar la novedad y nivel inventivo resultan equivalentes a los que la Superintendencia debe emplear. Tan cierto es lo anterior, que el Manual Andino de Patentes, al comentar el Artículo 46 de la Decisión 486, establece que una Oficina nacional competente podrá reconocer los resultados de exámenes de novedad o de patentabilidad efectuados respecto a la solicitud extranjera equivalente, como suficientes para acreditar el cumplimiento de las condiciones de patentabilidad.³ En el mismo sentido, es importante anotar que el mismo Tribunal Andino de Justicia ha citado a la jurisprudencia de las Cámaras de Apelación de Oficina Europea de Patentes como antecedentes aplicables a la Decisión 486.⁴

Así las cosas, deseo poner bajo su conocimiento el hecho de que en relación con la presente solicitud han sido otorgados diversos privilegios de patente a través del mundo entero, como por ejemplo en Australia, China y Nueva Zelanda, que protegen materia equivalente a la que reclama la presente solicitud colombiana. De nuevo, aunque dichas patentes no son vinculantes para la

³ Manual Andino de Patentes. Sección 7.3.

⁴ Ver por ejemplo:

- Proceso 193-IP-2005, página 12, donde se cita que “el Tribunal a manera de orientación ha tomado como referencia criterios de las Cámaras de Recursos de la Oficina Europea de Patentes (OEP)” para el análisis de la falta de novedad.
- Proceso No. 216-IP-2003, página 11, donde se menciona a manera de referencia la interpretación y el alcance que dan las directivas de la Oficina Europea de Patentes al término terapia.
- Proceso No. 118-IP-2005, página 15, donde el Tribunal cita a manera de referencia que “a partir de la “decisión adoptada en el caso T-506/95, las Cámaras han establecido que el estado de la técnica más próximo es aquel que...”.
- Proceso 69-IP-2005, página 12, donde el Tribunal explica que “para analizar la existencia de nivel inventivo, las Cámaras de Recursos han adoptado el método llamado de “acercamiento problema-solución” (problem and solution approach)”.
- Proceso No. 101-IP-2005, página 9, donde el Tribunal cita “a título de referencia, el criterio de las Cámaras de Recursos, según el cual los manuales u obras básicas deben formar parte de los conocimientos generales del experto medio”.

oficina nacional, si corresponden a un fuerte indicio sobre el cumplimiento de los requisitos de patentabilidad de la invención reivindicada, puesto que los requisitos de novedad, nivel inventivo son requisitos universales y objetivos.

6. Por todo lo anterior, considero que las objeciones presentadas por el Examinador han sido resueltas y por consiguiente solicito respetuosamente que se proceda a otorgar el privilegio de patente a la presente invención. En el caso que el Examinador considere que aún existen reivindicaciones no patentables, respetuosamente solicito emitir un segundo Auto de Fondo para hacer las aclaraciones correspondientes.

Atentamente,



J. IAN RAISBECK

C.C. 79'376.996 de Bogotá-D.C.

T.P. 135.799 del C. S. de la J.

Anexos:

- Capítulo Reivindicatorio Modificado.
- Recibo de pago correspondiente a la tasa de modificación.
- Sustitución de poder.
- Anexo 1: Sinergia entre las respuestas inmunes dirigidas contra la PhtD y el polisacárido capsular neumocócico en el *S. pneumoniae* en el modelo letal de ratón.
- Anexo 2: Ejemplos 3 a 8 relacionados con la presente invención.

CIENCIA DE PRESENTACION PERSONAL Y
RECONOCIMIENTO DE CONTENIDO Y FIRMA.
NOTARIA EL ANTERIOR ESCRITO DIRIGIDO A:
27 FUE PRESENTADO PERSONALMENTE
ANTE EL SEÑOR NOTARIO VEINTISIETE
DE SAN JUAN DE BOGOTÁ D.C.
Con C.C. 79376990
Y ADEMAS QUE EL NOTARIO SE
DIO CUENTA DE QUE LA FIRMA QUE LE
AUTORIZA FUE PUESTA POR EL
EL DECLARANTE:
Hoy: 02 OCT 2007 HUELLA
MARIA EUGENIA ROJAS DE URUETA
NOTARIA VEINTISIETE
REPUBLICA DE COLOMBIA

REPUBLICA DE COLOMBIA
MARIA EUGENIA ROJAS DE URUETA
NOTARIA
Notaria 27 Bogotá D.C.

Comerio
lan 2007
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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 07 - 75,614
FECHA : SEPTIEMBRE 18 DE 2007

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
OLARTE RAISBEK	CONSIGNACION	BANCO POPULAR	050-00110-6	68290537	18/09/2007	7,259,000.00

***** CONCEPTO *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-03	CAMBIO DE MODALIDAD Y MODIFICA	
		12 MARCAS Y LEMAS COMERCIALES	99,000.00
		TOTAL :	\$ 99,000.00

SON: NOVENTA Y NUEVE MIL PESOS

RESPONSABLE : _____

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

CAPÍTULO REIVINDICATORIO MODIFICADO

(examen de fondo)

1. Una composición inmunogénica que comprende derivados polisacáridos antigénicos de *Streptococcus pneumoniae* de al menos cuatro serotipos seleccionados del grupo que consiste en los serotipos 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F y 33F, y una proteína antigénica de *Streptococcus pneumoniae* seleccionada del grupo que consiste en PhtA, PhtB, PhtD y PhtE.

2. La composición inmunogénica de la Reivindicación 1, donde los antígenos polisacáridos se presentan en la forma de un conjugado polisacárido-proteína portadora.

3. La composición inmunogénica de la Reivindicación 2, donde la proteína portadora se selecciona del grupo que consiste en: toxoide diftérico, toxoide tetánico, CRM197, hemocianina de Megathura crenulata -lapa californiana- (keyhole Limpet Haemocyanin (KLH)), proteína derivada de tuberculina (PPD), y proteína D de *H. influenzae*.

4. Una composición inmunogénica de cualquiera de las Reivindicaciones 1 a 3, donde la vacuna comprende al menos siete polisacáridos antigénicos neumocócicos de diferentes serotipos.

5. Una composición inmunogénica de cualquiera de las Reivindicaciones 1 a 4, que comprende un adyuvante que comprende una sal de aluminio, por ejemplo gel de hidróxido de aluminio o fosfato de aluminio.

6. Una composición inmunogénica de cualquiera de las Reivindicaciones 1 a 5, que comprende un adyuvante que es preferiblemente un inductor de una respuesta TH1 y comprende uno, dos o tres de los siguientes: 3D-MPL, una saponina inmunoestimulante, o un oligonucleótido CpG inmunoestimulante.

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7. Una composición inmunogénica de la Reivindicación 6, donde el adyuvante comprende un portador seleccionado del grupo que comprende: una emulsión aceite en agua, liposomas, y una sal de aluminio.

8. Una vacuna que comprende la composición inmunogénica de las Reivindicaciones 1-7.

9. Un método para fabricar una composición inmunogénica como las aquí reivindicadas, que comprende los pasos de:

seleccionar uno o más polisacáridos antigénicos neumocócicos;

seleccionar una o más proteínas antigénicas neumocócicas del grupo que consiste en PhtA, PhtB, PhtD y PhtE; y

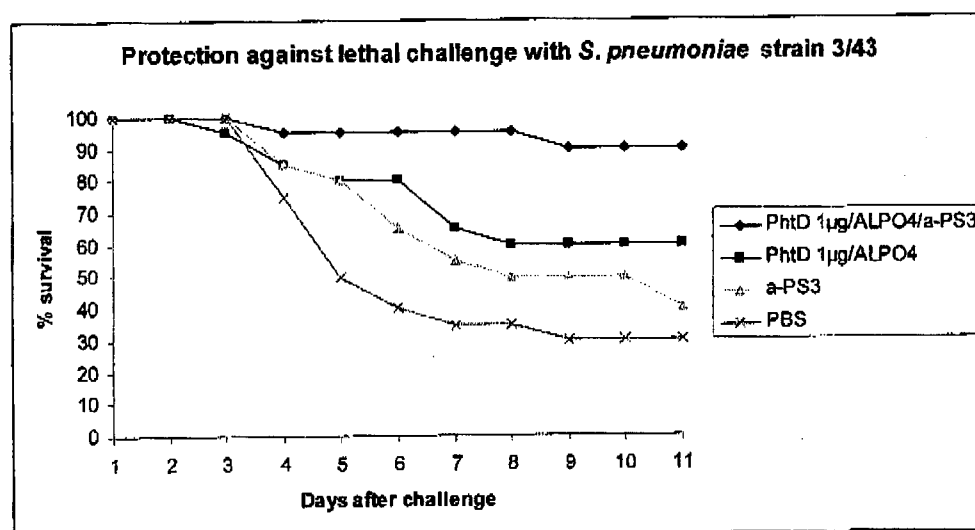
mezclar dichos polisacáridos y proteínas con un excipiente adecuado.

ANNEX 11) Synergy between immune responses directed against PhtD and pneumococcal capsular polysaccharide in the *S. pneumoniae* lethality mouse model.

The potential synergy between immune responses directed against PhtD and capsular polysaccharides was evaluated in the mouse lethal *S. pneumoniae* challenge model. Mice were intramuscularly immunized three times (D0, 14 and 28) with PhtD. One hour before the bacterial challenge, anti-polysaccharides antibodies were passively transferred to mice (IP, 200 μ l). Lethality induced by *S. pneumoniae* was followed up to 8 or 11 days after challenge. The synergy of protection is presented here for two *S. pneumoniae* strains (serotype 3 and serotype 1).

S. pneumoniae strain 3/43 challenge model:

In this experiment (LIMS 20040402), OF1 mice were immunized with PhtD adsorbed onto ALPO4 and 1.25 μ g of anti-PS3 guinea pig antibodies were passively transferred 1 hour before challenge with *S. pneumoniae* serotype 3 (Spn 3/43).

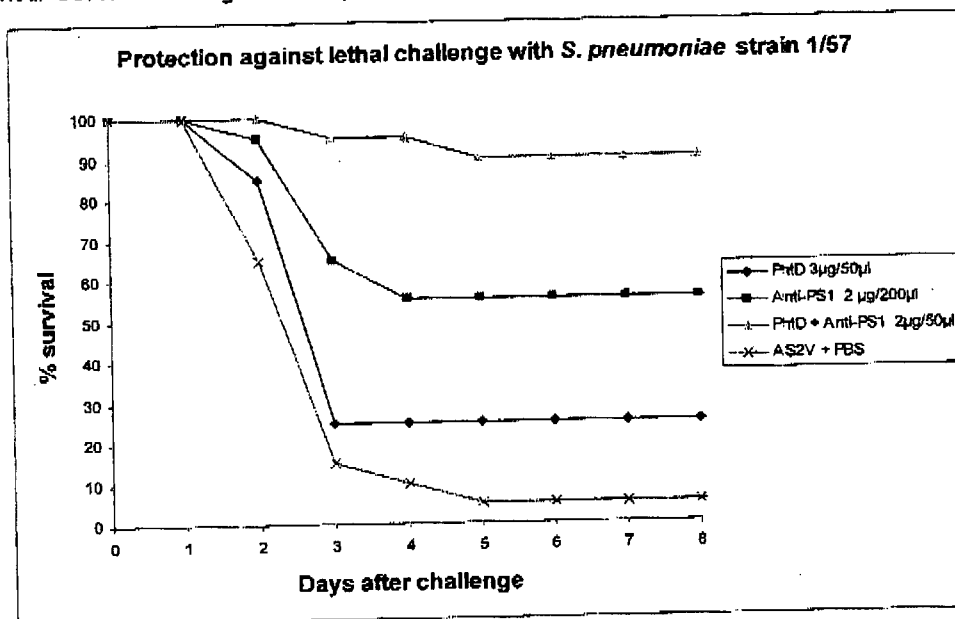


70% lethality was observed in mice having received PBS only. A moderate protection was observed in group of mice that received anti-PS3 antibodies or mice immunized with PhtD. A synergy, leading to an almost complete protection, was obtained in mice that combined active and passive immunization against PhtD and PS3, respectively.

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***S. pneumoniae* serotype 1/57 challenge model:**

In this experiment (LIMS 20070331), OF1 mice were immunized with PhtD adjuvanted with a TH1 adjuvant and anti-PS1 guinea pig antibodies were passively transferred one hour before challenge with *S. pneumoniae* serotype 1 (Spn 1/57).



A high lethality was observed in the control group in which mice received TH1 adjuvant (active immunization) and PBS only (passive immunization). A moderate protection was observed in group of mice that received anti-PS1 antibodies (55% survival) or mice immunized with PhtD (25% survival). A synergy, leading to an almost complete protection, was obtained in mice that combined active and passive immunization against PhtD and PS1, respectively.

Those data support the synergistic effect of the immune responses directed against a pneumococcal protein (i. e. PhtD) and capsular polysaccharides in the mechanism of protection against *S. pneumoniae* infection.

Annex 2

Example 3, Effectiveness of Pneumococcal Histidine Triad Protein D (PhtD) as a protein carrier to enhance the immunogenicity of PS 22F in young Balb/c mice

5 Groups of 40 female Balb/c mice (4-weeks old) were immunized IM at days 0, 14 and 28 with 50 µl of either 4-valent plain PS or 4-valent PhtD-conjugated PS, both admixed with Adjuvant C.

10 Both vaccine formulations were composed of 0.1 µg (quantity of saccharide) of each of the following PS: PS8, PS12F, PS19F and PS22F.

Anti-PS ELISA IgG levels were dosed in sera collected at day 42.

The anti-PS22F response, shown as an example in Figure 4, was strongly enhanced in mice given 4-valent PhtD conjugates compared to mice immunized with the plain PS. The same improvement was observed for the anti-PS8, 12F and 19F IgG responses (data not shown).

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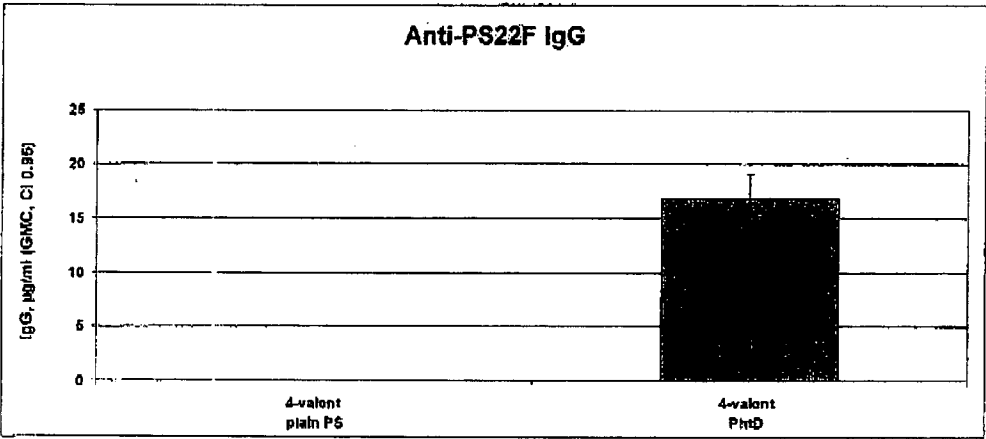


Figure 1 PS22F immunogenicity in Balb/c mice (post-III IgG levels)

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Example 4, Immunogenicity in elderly C57Bl mice of 13-valent PS conjugates containing 19A-dPly and 22F-PhtD

5 Groups of 30 old C57Bl mice (>69-weeks old) were immunized IM at days 0, 14 and 28 with 50 µl of either 11-valent PS conjugates or 13-valent PS conjugates, both admixed with Adjuvant C (see below).

10 The 11-valent vaccine formulation was composed of 0.1 µg saccharide of each of the following conjugates: PS1-PD, PS3-PD, PS4-PD, PS5-PD, PS6B-PD, PS7F-PD, PS9V-PD, PS14-PD, PS18C-TT, PS19F-DT and PS23F-PD. The 13-valent vaccine formulation contained in addition 0.1 µg of PS19A-dPly and PS22F-PhtD conjugates. In group 2 and 4 the pneumolysin carrier was detoxified with GMBS treatment, in group 3 and 5 it was done with formaldehyde. In groups 2 and 3 PhtD was used to conjugate PS 22F, in Groups 4 and 5 a PhtD_E fusion (the construct VP147 from WO 03/054007) was used. In group 6 19A was conjugated to diphtheria toxoid and 22F to protein D.

15 Anti-PS19A and 22F ELISA IgG levels were dosed in individual sera collected at day 42. The ELISA IgG response generated to the other PS was measured in pooled sera.

20 19A-dPly and 22F-PhtD administered within the 13-valent conjugate vaccine formulation were shown immunogenic in old C57Bl mice (Table 15). The immune response induced against the other PS was not negatively impacted in mice given the 13-valent formulation compared to those immunized with the 11-valent formulation.

Table 1 PS immunogenicity in old C57Bl mice (post-III IgG levels)

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Old C57 Black mice						
ELISA	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
	11V 0.1µg/50µl Adj C	11V 19A-dPly gmbs 22F-PhtD 0.1µg/50µl Adj C	11V 19A-dPly formol 22F-PhtD 0.1µg/50µl Adj C	11V 19A-dPly gmbs 22F-PhtD-E 0.1µg/50µl Adj C	11V 19A-dPly formol 22F-PhtD-E 0.1µg/50µl Adj C	11V 19A-DT 22F-PD 0.1µg/50µl Adj C
1 average Pool	19.30	20.20	24.40	12.80	12.10	13.60
3 average Pool	6.32	4.84	5.21	6.74	2.38	2.54
4 average Pool	60.9	67.1	51.4	47.4	45.5	41.1

5	average Pool	1.34	3.81	3.06	2.75	1.26	1.23
6B	average Pool	4.41	4.12	5.88	1.58	2.31	5.64
7F	average Pool	0.83	0.81	1.65	1.98	0.89	0.99
9V	average Pool	13.8	23.7	20.0	13.1	15.5	9.6
14	average Pool	25.73	42.96	34.12	32.53	23.97	15.60
18C	average Pool	13.4	20.1	11.9	9.1	8.3	8.4
19F	average Pool	57.5	90.0	63.8	36.5	47.0	69.1
23F	average Pool	NR	NR	NR	NR	NR	NR
19A	GMC IC %sero	0.06 0.04-0.1 33%	0.09 0.05-0.14 47%	0.25 0.15-0.41 83%	0.08 0.06-0.12 53%	0.23 0.14-0.38 80%	0.19 0.09-0.3 73%
22F	GMC IC %sero	NR 0%	5.81 3.2-10.6 97%	3.76 1.8-7.9 90%	0.54 0.3-1.1 77%	0.85 0.4-1.7 87%	2.02 1.2-3.4 97%

Example 5, Immunogenicity in young Balb/c mice of 13-valent PS conjugates containing 19A-dPly and 22F-PhtD

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Groups of 30 young Balb/c mice (4-weeks old) were immunized IM at days 0, 14 and 28 with 50 µl of either 11-valent PS conjugates or 13-valent PS conjugates, both admixed with Adjuvant C (see below).

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The 11-valent vaccine formulation was composed of 0.1 µg saccharide of each of the following conjugates: PS1-PD, PS3-PD, PS4-PD, PS5-PD, PS6B-PD, PS7F-PD, PS9V-PD, PS14-PD, PS18C-TT, PS19F-DT and PS23F-PD (see Table 1 and comment on 11 valent vaccine discussed under Table 2). The 13-valent vaccine formulation contained in addition 0.1 µg of PS19A-dPly and PS22F-PhtD conjugates (see Table 1 and comment on 13 valent vaccine discussed under Table 2 [using directly-conjugated 22F]). In group 2

15

and 4 the pneumolysin carrier was detoxified with GMBS treatment, in group 3 and 5 it

was done with formaldehyde. In groups 2 and 3 PhtD was used to conjugate PS 22F, in Groups 4 and 5 a PhtD_E fusion (the construct VP147 from WO 03/054007) was used. In group 6 19A was conjugated to diphtheria toxoid and 22F to protein D.

Anti-PS19A and 22F ELISA IgG levels were dosed in individual sera collected at day 42.

- 5 The ELISA IgG response generated to the other PS was measured in pooled sera. 19A-dPly and 22F-PhtD administered within the 13-valent conjugate vaccine formulation were shown immunogenic in young Balb/c mice (Table 16). The immune response induced against the other PS was not negatively impacted in mice given the 13-valent formulation compared to those immunized with the 11-valent formulation.

10

Table 2, PS immunogenicity in young Balb/c mice (post-III IgG levels)

BalbC mice						
ELISA	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
	11V	11V	11V	11V	11V	11V
		19A-dPly	19A-dPly	19A-dPly	19A-dPly	19A-DT
		gmbs	formol	gmbs	formol	22F-PD
		22F-PhtD	22F-PhtD	22F-PhtD-E	22F-PhtD-E	
	0.1µg/50µl	0.1µg/50µl	0.1µg/50µl	0.1µg/50µl	0.1µg/50µl	0.1µg/50µl
	Adj C	Adj C	Adj C	Adj C	Adj C	Adj C
1 average Pool	131.70	101.20	83.00	82.40	67.90	85.50
3 average Pool	21.85	10.38	12.53	8.83	8.73	14.98
4 average Pool	147.4	127.0	104.4	95.0	113.6	114.2
5 average Pool	21.38	20.29	18.26	18.95	18.02	23.04
6B average Pool	1.97	4.76	3.72	2.35	1.43	1.05
7F average Pool	7.69	4.58	4.77	4.24	3.92	3.94
9V average Pool	30.1	30.7	26.5	21.4	23.4	28.3
14 average	28.78	27.67	26.23	21.54	24.34	13.73

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Pool							
18C	average Pool	53.4	52.37	46.5	57.8	47.8	75.8
19F	average Pool	186.6	157.7	169.3	178.9	181.9	223.2
23F	average Pool	4.98	3.9	5.11	0.57	3.13	4.57
19A	GMC	0.4	32.8	25.1	21.6	18.9	23.5
	IC	0.2-0.6	26.4-40.7	20.6-30.6	17.5-26.7	15.1-23.5	19.5-28.5
	%sero	93%	100%	100%	100%	100%	100%
22F	GMC	NR	3.99	3.76	6.27	8.70	18.76
	IC		1.9-8.42	1.8-8	3.8-10.4	5.4-13.9	15.2-23.1
	%sero	0%	93%	100%	100%	100%	100%

Example 6, Immunogenicity in Guinea Pigs of 13-valent PS conjugates containing 19A-dPly and 22F-PhtD

- 5 Groups of 20 young Guinea Pigs (Hartley Strain; 5 weeks old) were immunized IM at days 0, 14 and 28 with 125 µl of either 11-valent PS conjugates or 13-valent PS conjugates, both admixed with Adjuvant C (see below).
- The 11-valent vaccine formulation was composed of 0.25 µg saccharide of each of the following conjugates: PS1-PD, PS3-PD, PS4-PD, PS5-PD, PS6B-PD, PS7F-PD, PS9V-PD, PS14-PD, PS18C-TT, PS19F-DT and PS23F-PD (see Table 1 and comment on 11
- 10 valent vaccine discussed under Table 2). The 13-valent vaccine formulation contained in addition 0.1 µg of PS19A-dPly and PS22F-PhtD conjugates (see Table 1 and comment on 13 valent vaccine discussed under Table 2 [using directly-conjugated 22F]). In group 2 and 4 the pneumolysin carrier was detoxified with GMBS treatment, in group 3 and 5 it
- 15 was done with formaldehyde. In groups 2 and 3 PhtD was used to conjugate PS 22F, in Groups 4 and 5 a PhtD_E fusion (the construct VP147 from WO 03/054007) was used. In group 6 19A was conjugated to diphtheria toxoid and 22F to protein D.
- Anti-PS19A and 22F ELISA IgG levels were dosed in individual sera collected at day 42. The ELISA IgG response generated to the other PS was measured in pooled sera.

Table 3, PS immunogenicity in young Balb/c mice (post-III IgG levels)

Guinea pigs						
ELISA	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
	11V	11V	11V	11V	11V	11V

19

		0.1µg/50µl Adj C	19A-dPly gmbs 22F-PhtD 0.1µg/50µl Adj C	19A-dPly formol 22F-PhtD 0.1µg/50µl Adj C	19A-dPly gmbs 22F-PhtD- E 0.1µg/50µl Adj C	19A-dPly formol 22F-PhtD-E 0.1µg/50µl Adj C	19A-DT 22F-PD 0.1µg/50µl Adj C
1	average Pool	78.00	77.21	76.15	68.77	68.59	81.04
3	average Pool	7.75	9.31	12.73	7.94	4.75	9.59
4	average Pool	130.7	94.4	132.6	166.8	85.0	101.3
5	average Pool	109.10	117.10	110.70	158.40	74.10	100.40
6B	average Pool	3.14	4.26	14.4	7.63	6.3	7.52
7F	average Pool	154.2	216.0	240.0	181.0	142.0	179.1
9V	average Pool	90.69	105.45	98.20	93.45	54.12	73.05
14	average Pool	71.19	77.18	46.53	59.67	38.47	53.69
18C	average Pool	109.4	122.3	137.1	79.9	73.7	83.1
19F	average Pool	73.9	102.5	112.2	75.5	62.3	72.1
23F	average Pool	19.19	30.74	29.44	31.52	19.13	24.94
19A	GMC	0.4	25.58	41.49	14.25	27.49	6.74
	IC	0.24-0.68	12-54.5	24.4-70.5	5.9-34.6	16.6-45.4	4-11.3
	%sero	75%	100%	100%	100%	100%	100%
22F	GMC	0.12	2.51	3.67	45.74	30.68	96.38
	IC	0.09-0.16	0.94-6.73	1.59-8.42	29.3-71.4	17-53.3	73.5-126.4
	%sero	10%	95%	95%	100%	100%	100%

20

Example 7: Formulations being made and tested

- 5 a) The following formulations are made (using the 13 valent vaccine from table 1 and serotype 3 from table 5 – see comment on 14 valent vaccine discussed under Table 2 [using directly-conjugated 22F or through an ADH linker]). The saccharides are formulated with aluminium phosphate and 3D-MPL as shown below.

14V 25µg MPL Sum of BAC Aluminium content -> FF Per Dose:						14V 10µg MPL Sum of BAC Aluminium content -> FF Per Dose:					
PS	carrier	µg PS	µg MPL	ratio PS/Al 1/x	µg Al	PS	carrier	µg PS	µg MPL	ratio PS/Al 1/x	µg Al
1	PD	1		10	10	1	PD	1		10	10
3	PD	1		10	10	3	PD	1		10	10
4	PD	3		10	30	4	PD	3		10	30
5	PD	1		10	10	5	PD	1		10	10
6A	PD	1		10	10	6A	PD	1		10	10
6B	PD	1		10	10	6B	PD	1		10	10
7F	PD	1		10	10	7F	PD	1		10	10
9V	PD	1		10	10	9V	PD	1		10	10
14	PD	1		10	10	14	PD	1		10	10
18C	TT _{AD}	3		15	45	18C	TT _{AD}	3		15	45
19A	dPly	3		10	30	19A	dPly	3		10	30
19F	DT	3		10	30	19F	DT	3		10	30
22F	PhD	3		10	30	22F	PhD	3		10	30
23F	PD	1		10	10	23F	PD	1		10	10
BAC MPL 50/200			25	4	100	BAC MPL 50/200			10	4	40
FF Aluminium content				Sum =	355	FF Aluminium content				Sum =	295

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- b) The same saccharide formulation is adjuvanted with each of the following adjuvants:

- 15 - In the table herebelow the concentration of the emulsion components per 500µl dose is shown.

	Adjuvant A1	Adjuvant A2	Adjuvant A3
Ingredients	250µl o/w emulsion	125µl o/w emulsion	50µl o/w emulsion
alpha	11.88mg	5.94mg	2.38mg
Tocopherol			
Squalene	10.7mg	5.35mg	2.14mg
Tween 80	4.85mg	2.43mg	0.97mg

	Adjuvant A4	Adjuvant A5	Adjuvant A6	Adjuvant A7
Ingredients	250µl o/w emulsion	250µl o/w emulsion	125µl o/w emulsion	50µl o/w emulsion
alpha	11.88mg	11.88mg	5.94mg	2.38mg
Tocopherol				
Squalene	10.7mg	10.7mg	5.35mg	2.14mg
Tween 80	4.85mg	4.85mg	2.43mg	0.97mg
3D-MPL	50µg	25µg	25µg	10µg

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c) The saccharides are also formulated with two liposome based adjuvants:

Composition of Adjuvant B1

5 Qualitative Quantitative (per 0.5 mL dose)

Liposomes:

- DOPC 1 mg
- cholesterol 0.25 mg

3DMPL 50 µg

10 QS21 50 µg

KH₂PO₄ 13.124 mg Buffer

Na₂HPO₄ 0.290 mg Buffer

NaCl 2.922 mg
(100 mM)

15 WFI q.s. ad 0.5 ml Solvent

pH 6.1

1. Total PO₄ concentration = 50 mM

Composition of Adjuvant B2

20 Qualitative Quantitative (per 0.5 mL dose)

Liposomes:

- DOPC 0.5 mg
- cholesterol 0.125 mg

3DMPL 25 µg

25 QS21 25 µg

KH₂PO₄ 13.124 mg Buffer

Na₂HPO₄ 0.290 mg Buffer

NaCl 2.922 mg
(100 mM)

30 WFI q.s. ad 0.5 ml Solvent

pH 6.1

d) The saccharides are also formulated with Adjuvant C (see above for other compositions where this adjuvant has been used):

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Qualitative Quantitative (per 0.5 mL dose)

Oil in water emulsion: 50 µl

- squalene 2.136 mg
- α-tocopherol 2.372 mg

40 - Tween 80 0.97 mg

- cholesterol 0.1 mg

3DMPL 50 µg

QS21 50 µg

KH₂PO₄ 0.470 mg Buffer

45 Na₂HPO₄ 0.219 mg Buffer

NaCl 4.003 mg
(137 mM)

KCl 0.101 mg
(2.7 mM)
WFI q.s. ad 0.5 ml Solvent
pH 6.8

5

Example 8. impact of new adjuvants on immunogenicity of *Streptococcus pneumoniae* capsule PS conjugates

- 10 Groups of 40 female Balb/c mice were immunised by the IM route at days 0, 14 and 28 with 13-valent PS formulations containing PS 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F and 23F (dose: 0.3 µg / conjugate for PS 4, 18C, 19A, 19F and 22F and 0.1 µg / conjugate for the other PS).

- 15 PS 18C was conjugated to Tetanus Toxoid, 19F to Diphtheria Toxoid, 19A to formol-detoxified Ply, 22F to PhtD and the other PS to PD.

Four formulations, supplemented with either AlPO₄, adjuvant A1, adjuvant A4 or adjuvant A5, were compared.

- 20 Anti-PS, Ply, PhtD and PD ELISA IgG levels were measured in sera collected at day 42 and pooled per group. The following ratio was calculated for each antigen: IgG level induced with the new adjuvant tested / IgG level induced with AlPO₄.

All the new adjuvants tested improved at least 2-fold the immune responses to 13-valent conjugates compared to the classical AlPO₄ formulation (figure 2).

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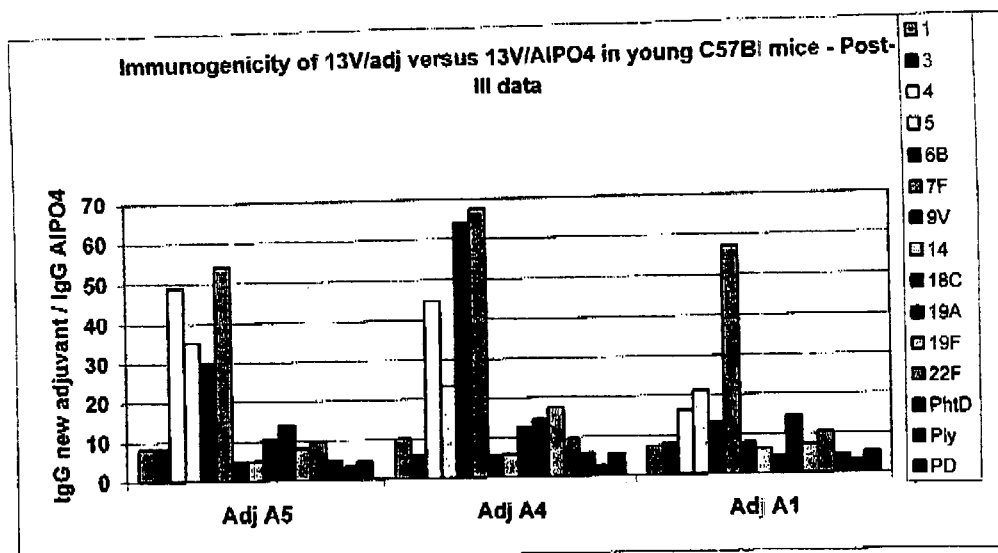


Figure 2, comparison of IgG responses induced with new adjuvants to the response elicited with AlPO₄

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C07K 13/00, C12P 21/00 C12N 15/31, C07H 21/04	A1	(11) International Publication Number: WO 90/06951 (43) International Publication Date: 28 June 1990 (28.06.90)
(21) International Application Number: PCT/AU89/00539 (22) International Filing Date: 15 December 1989 (15.12.89) (30) Priority data: PJ 1989 16 December 1988 (16.12.88) AU (71)(72) Applicants and Inventors: PATON, James, Cleland [AU/AU]; 49 Foster Street, Parkside, S.A. 5063 (AU). HANSMAN, David, John [AU/AU]; 66 Alexandra Avenue, Rose Park, S.A. 5067 (AU). BOULNOIS, Graham, John [GB/GB]; 26 Prospect Road, Kibworth Beauchamp, Leicestershire LE1 9HN (GB). ANDREW, Peter, William [GB/GB]; 7 Chapel Lane, Leister, Leicestershire LE1 9HN (GB). MITCHELL, Timothy, John [GB/GB]; 25 Mawbys Lane, Appleby Magna, Burton-on-Trent, Staffordshire DE12 7AA (GB). WALKER, John, Arthur [GB/US]; Traymore Apts., No. 11, 51 S. Mclean, Memphis, TN 38104 (US).	(74) Agent: COLLISON & CO.; 117 King William Street, Adelaide, S.A. 5000 (AU). (81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE, DE (European patent), DK, ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US. Published <i>With international search report.</i>	
(54) Title: PNEUMOLYSIN MUTANTS AND PNEUMOCOCCAL VACCINES MADE THEREFROM (57) Abstract Mutants of pneumolysin that are non-toxic by reason of amino acid substitutions have been constructed. These mutants elicit an immune response in animals that is reactive to wild-type pneumolysin. The invention also encompasses vaccines for humans based on these mutants, including vaccines comprising conjugates with pneumococcal capsular polysaccharides.		

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PNEUMOLYSIN MUTANTS AND PNEUMOCOCCAL VACCINES
MADE THEREFROM

5 This invention relates to mutants of the toxin pneumolysin and pneumococcal vaccines based on these mutants.

BACKGROUND

10 *Streptococcus pneumoniae* (pneumococcus) is an important pathogen, causing invasive diseases such as pneumonia, meningitis and bacteraemia. Even in regions where effective antibiotic therapy is freely available, the mortality rate from pneumococcal pneumonia can be as high as 19% in hospitalized patients and this increases to 30-40% in patients with bacteraemia. These high mortality rates have been reported in the U.S.A. where pneumonia, of which *S. pneumoniae* is the commonest cause, is the fifth ranking cause of death. Indeed, pneumonia is the only infectious disease amongst the top ten causes of death in that country. In the United States mortality rates for pneumococcal meningitis range from 13-45%. In developing countries, in excess of 3 million children under the age of 5 years die each year from pneumonia, and again *S. pneumoniae* is the commonest causative agent. *S. pneumoniae* also causes less serious, but highly prevalent infections such as otitis media and sinusitis, which have a significant impact on health-care costs in developed countries. Otitis media is especially important in young children; sinusitis affects both children and adults.

25 In the late 1970's, a vaccine was licensed for the purpose of preventing serious infections, especially bacterial pneumonia and for protecting certain groups, such as splenectomized individuals and young children, who are particularly susceptible to fulminating pneumococcal disease. The vaccine is composed of purified capsular polysaccharides, which are the predominant pneumococcal surface antigens. However, each serotype of *S. pneumoniae* (of which there are 83) has a structurally distinct capsular polysaccharide, and immunization with one serotype confers no protection whatsoever against the vast majority of the others. The vaccine currently licensed in Australia contains polysaccharides purified from the 23 most common serotypes, which account for approximately 90% of pneumococcal infections in this country.

Protection even against those serotypes contained in the vaccine is by no means complete, and there have been several reports of serious, even fatal infections occurring in vaccinated high-risk individuals. The efficacy of the vaccine is poorest in young children, and several studies, including one conducted in Adelaide, have shown that the existing formulation has little or no demonstrable clinical benefit in this group. This apparent failure of the vaccine appears to be related to the poor immunogenicity of certain pneumococcal polysaccharides in children under 5 years of age. We have shown that the antibody response is particularly poor to the five serotypes which most commonly cause disease in children (types 6, 14, 18, 19 and 23). Indeed, the antibody response to these pneumococcal polysaccharides only approaches adult levels in children over 8 years of age at the time of vaccination.

In view of this, a vaccine, including antigens other than the capsular polysaccharides seems to be required to protect young children from pneumococcal infection. One such antigen could be pneumolysin, a protein toxin produced by all virulent *S. pneumoniae* isolates. Immunization of mice with this protein has been found to confer a degree of protection from pneumococcal infection.

However there is a difficulty in that pneumolysin is toxic to humans. Thus pneumolysin included in a vaccine must therefore be substantially non-toxic. However, the rendering of a pneumolysin non-toxic by most currently employed methods would be likely to alter the basic configuration of the protein so as to be immunogenically distinct from the native or wild-type pneumolysin. An immune response elicited by an altered protein that is immunogenically distinct from the native pneumolysin will have a decreased protective capacity or no protective capacity. Thus the difficulty is to produce an altered pneumolysin that is non-toxic and at the same time sufficiently immunogenically similar to the toxic form to elicit a protective immune response.

An altered pneumolysin with the above characteristics can then be used in a number of ways in a vaccine. Thus the altered pneumolysin may be used by itself to immunise, or alternatively the altered pneumolysin may be conjugated to pneumococcal polysaccharide, or

alternatively may be included in a vaccine wherein pneumococcal polysaccharides may be conjugated to another protein and the altered pneumolysin is present in a non-conjugated form only. Alternatively, pneumococcal polysaccharide and pneumolysin may both be used in an unconjugated form.

DESCRIPTION OF INVENTION

In a broad form therefore the invention may be said to reside in an altered pneumolysin being substantially non-toxic and being capable of eliciting an immune response in an animal being reactive to wild-type pneumolysin.

Preferably the altered pneumolysin has reduced complement binding activity as compared to wild-type pneumolysin. Reduction in the complement binding activity results in less inflammation at the site of administering the vaccine.

Preferably the altered pneumolysin has reduced Fc binding activity as compared to wild-type pneumolysin. Reduction in the Fc binding activity results in less inflammation at the site of administering the vaccine.

Preferably the altered pneumolysin is altered by reason of one or more amino acid substitutions relative to wild-type pneumolysin.

The pneumolysin may be altered in that the amino acid present at any one or more than one of residue sites 367, 384, 385, 428, 433 or 435 of wild-type pneumolysin are replaced, removed or blocked.

In a further form the invention could be said to reside in a vaccine including an altered pneumolysin, said altered pneumolysin being non-toxic and being capable of eliciting an immune response in an animal being reactive to wild-type pneumolysin.

Preferably the vaccine comprises capsular polysaccharide material conjugated with the altered pneumolysin.

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The capsular material may be derived from any one or more of the *Streptococcus pneumoniae* serotypes 6A, 6B, 14, 18C, 19A, 19F, 23F, 1, 2, 3, 4, 5, 7F, 8, 9N, 9V, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F.

- 5 In this embodiment serotypes which are commonly associated with disease in children, and to which children generally have a poor immune response, may be specifically targeted (i.e. Danish serotypes 6A, 6B, 14, 18C, 19A, 19F and 23F). Other common serotypes contained in the present 23-valent Merck Sharp and Dohme vaccine
10 (Pneumovax 23) however, could also be used to synthesize conjugates (i.e. types 1, 2, 3, 4, 5, 7F, 8, 9N, 9V, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F) or indeed any other serotype. Conjugation of any pneumococcal polysaccharides to the protein carrier ensures good T-cell dependent immunogenicity in children, such that protective levels
15 of anti-polysaccharide antibody are produced.

The combination of the altered pneumolysin together with the capsular material will ensure an extra degree of protection, particularly against serotypes of *S. pneumoniae* whose polysaccharides are not
20 incorporated in the existing vaccine formulations.

The vaccine is preferably administered by sub-cutaneous injection, with or without an approved adjuvant, such as alumina gel.

- 25 In another form the invention could be said to reside in a recombinant clone including a replicon and a DNA sequence encoding an altered pneumolysin, said altered pneumolysin being non-toxic and being capable of eliciting an immune response in an animal being reactive to wild-type pneumolysin.

30 In yet another form the invention could be said to reside in a method of producing an altered pneumolysin including the steps of purifying said altered pneumolysin from an expression system including a recombinant clone with DNA encoding an altered pneumolysin said
35 pneumolysin being substantially non-toxic and being capable of eliciting an immune response in an animal reactive to wild-type pneumolysin.

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Preferrably the expression system is a culture of a host cell including a recombinant clone with DNA encoding the altered pneumolysin.

5 In another form the invention could be said to reside in a method of producing a vaccine including the step of amplifying a recombinant clone encoding an altered pneumolysin, inducing transcription and translation of said cloned material, the purification of altered pneumolysin, and the step of conjugating the altered pneumolysin with a capsular polysaccharide, the altered pneumolysin having
10 substantially reduced toxic activity as compared with wild-type pneumolysin.

For a better understanding of the invention specific embodiments of the
15 invention will now be described with reference to diagrams wherein:-

FIG. 1 Is the DNA sequence of the gene encoding wild-type pneumolysin,

20 FIG. 2 Is the DNA sequence of an altered gene encoding wild type pneumolysin used for cloning the pneumolysin gene into an expression vector,

FIG. 3 Is the amino acid sequence of the wild-type pneumolysin as
25 derived from the DNA sequence of the gene encoding the wild type pneumolysin, and

FIG. 4 shows the amino acid sequence of pneumolysin showing amino acid substitutions introduced by site directed mutagenesis.
30

Recombinant DNA techniques have been used to construct non-toxic pneumolysin derivatives suitable for administration to humans. To achieve this, the *S. pneumoniae* gene encoding pneumolysin was
35 cloned into *Escherichia coli* and its complete DNA sequence determined. The DNA sequence is shown in Figure 1 and the derived amino acid sequence is shown in Figure 3.

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Three regions of the pneumolysin gene were subjected to oligonucleotide-directed mutagenesis. The first region encodes amino acids 427 - 437 in the protein sequence, and is indicated by an underline in Figure 3. This 11 amino acid sequence shows absolute
5 homology with similar regions in other related thiol activated toxins thus is thought to be responsible for the haemolytic activity and hence toxic activity of the toxin. The other two regions encode amino acids 257 - 297 and amino acids 368 - 397 and are also indicated by an underline in Figure 3. These two regions of the toxin have substantial
10 amino acid sequence homology with human C-reactive protein (CRP), and by inference therefore, are thought to be responsible for the ability of pneumolysin to bind the Fc region of immunoglobulins and to activate complement. Fifteen separate mutations in the pneumolysin gene, resulting in single amino acid substitutions, were constructed, as
15 shown in Figure 4. In an effort to maintain the structure of the altered pneumolysin, conservative substitutions were made, so that amino acids are substituted with amino acids of a similar nature.

For the region involved in haemolytic activity, Cys 428 -> Gly, Cys 428 -> Ser, Trp₄₃₃ -> Phe, Glu₄₃₄ -> Asp and Trp₄₃₅ -> Phe each reduced
20 haemolytic activity by 97%, 90%, 99%, 75% and 90% respectively. The other mutations in that region (Cys₄₂₈ -> Ala, Glu₄₃₄ -> Gln and Trp₄₃₆ -> Phe) did not affect haemolytic activity. Mutating a separate region of the toxin thought to be responsible for binding to target cell membranes
25 also affects haemolytic activity of the protein. This substitution, His₃₆₇ -> Arg, completely inhibits haemolytic activity. This is a quite unpredictable finding in that His₃₆₇ -> Arg therefore shows a greater inhibition of this property than the substitutions made within the 11 amino acid region thought to be responsible for haemolytic activity.

30 Mutations in the CRP-like domains were tested for ability to activate complement. For Trp₃₇₉ -> Phe, Tyr₃₈₄ -> Phe, Asp₃₈₅ -> Asn, and Trp₃₉₇ -> Phe, complement activation was reduced by 20%, 70%, 100% and 15%, respectively. The other mutations in the CRP-like
35 domains shown in Figure 4 do not reduce complement activation.

1. An altered pneumolysin being substantially non-toxic and being capable of eliciting an immune response in an animal being reactive to wild-type pneumolysin.

5 2. An altered pneumolysin as in claim one having reduced complement binding activity as compared to wild-type pneumolysin.

3. An altered pneumolysin as in any one of claims 1 or 2 having reduced Fc binding activity as compared to wild-type pneumolysin.

10

4. An altered pneumolysin as in any one of claims 1, 2, or 3 wherein said altered pneumolysin is altered by reason of one or more amino acid substitutions within wild type pneumolysin.

15 5. An altered pneumolysin having the following amino acid sequence:-

	Met	Ala	Asn	Lys	Ala	Val	Asn	Asp	Phe	Ile	Leu	Ala	Met
	1										11		
	Asn	Tyr	Asp	Lys	Lys	Lys	Leu	Leu	Thr	His	Gln	Gly	Glu
20							21						
	Ser	Ile	Glu	Asn	Arg	Phe	Ile	Lys	Glu	Gly	Asn	Gln	Leu
				31									
	Pro	Asp	Glu	Phe	Val	Val	Ile	Glu	Arg	Lys	Lys	Arg	Ser
	41										51		
25	Leu	Ser	Thr	Asn	Thr	Ser	Asp	Ile	Ser	Val	Thr	Ala	Thr
									61				
	Asn	Asp	Ser	Arg	Leu	Tyr	Pro	Gly	Ala	Leu	Leu	Val	Val
						71							
	Asp	Glu	Thr	Leu	Leu	Glu	Asn	Asn	Pro	Thr	Leu	Leu	Ala
30			81										91
	Val	Asp	Arg	Ala	Pro	Met	Thr	Tyr	Ser	Ile	Asp	Leu	Pro
									101				
	Gly	Leu	Ala	Ser	Ser	Asp	Ser	Phe	Leu	Gln	Val	Glu	Asp
							111						
35	Pro	Ser	Asn	Ser	Ser	Val	Arg	Gly	Ala	Val	Asn	Asp	Leu
				121									
	Leu	Ala	Lys	Trp	His	Gln	Asp	Tyr	Gly	Gln	Val	Asn	Asn
	131										141		
	Val	Pro	Ala	Arg	Met	Gln	Tyr	Glu	Lys	Ile	Thr	Ala	His
40								151					
	Ser	Met	Glu	Gln	Leu	Lys	Val	Lys	Phe	Gly	Ser	Asp	Phe
					161								
	Glu	Lys	Thr	Gly	Asn	Ser	Leu	Asp	Ile	Asp	Phe	Asn	Ser
	171											181	
45	Val	His	Ser	Gly	Glu	Lys	Gln	Ile	Gln	Ile	Val	Asn	Phe
									191				

	Lys	Gln	Ile	Tyr	Tyr	Thr	Val	Ser	Val	Asp	Ala	Val	Lys
						201							
5	Asn	Pro	Gly	Asp	Val	Phe	Gln	Asp	Thr	Val	Thr	Val	Glu
			211										221
	Asp	Leu	Lys	Gln	Arg	Gly	Ile	Ser	Ala	Glu	Arg	Pro	Leu
										231			
	Val	Tyr	Ile	Ser	Ser	Val	Ala	Tyr	Gly	Arg	Gln	Val	Tyr
						241							
10	Leu	Lys	Leu	Glu	Thr	Thr	Ser	Lys	Ser	Asp	Glu	Val	Glu
			251										
	Ala	Ala	Phe	Glu	Ala	Leu	Ile	Lys	Gly	Val	Lys	Val	Ala
	261									271			
15	Pro	Gln	Thr	Glu	Trp	Lys	Gln	Ile	Leu	Asp	Asn	Thr	Glu
							281						
	Val	Lys	Ala	Val	Ile	Leu	Gly	Gly	Asp	Pro	Ser	Ser	Gly
				291									
	Ala	Arg	Val	Val	Thr	Gly	Lys	Val	Asp	Met	Val	Glu	Asp
	301										311		
20	Leu	Ile	Gln	Glu	Gly	Ser	Arg	Phe	Thr	Ala	Asp	His	Pro
								321					
	Gly	Leu	Pro	Ile	Ser	Tyr	Thr	Thr	Ser	Phe	Leu	Arg	Asp
					331								
25	Asn	Val	Val	Ala	Thr	Phe	Gln	Asn	Ser	Thr	Asp	Tyr	Val
		341											351
	Glu	Thr	Lys	Val	Thr	Ala	Tyr	Arg	Asn	Gly	Asp	Leu	Leu
										361			
	Leu	Asp	R ₁	Ser	Gly	Ala	Tyr	Val	Ala	Gln	Tyr	Tyr	Ile
					371								
30	Thr	R ₂	Asp	Glu	Leu	Ser	R ₃	R ₄	His	Gln	Gly	Lys	Glu
			381										
	Val	Leu	Thr	Pro	Lys	Ala	R ₅	Asp	Arg	Asn	Gly	Gln	Asp
	391									401			
35	Leu	Thr	Ala	His	Phe	Thr	Thr	Ser	Ile	Pro	Leu	Lys	Gly
							411						
	Asn	Val	Arg	Asn	Leu	Ser	Val	Lys	Ile	Arg	Glu	R ₆	Thr
				421									
	Gly	Leu	Ala	R ₇	R ₈	R ₉	Trp	Arg	Thr	Val	Tyr	Glu	Lys
	431											441	
40	Thr	Asp	Leu	Pro	Leu	Val	Arg	Lys	Arg	Thr	Ile	Ser	Ile
							451						
	Trp	Gly	Thr	Thr	Leu	Tyr	Pro	Gln	Val	Glu	Asp	Lys	Val
					461								
45	Glu	Asn	Asp										
			471										

wherein R₁ is His or Arg, R₂ is Trp or Phe, R₃ is Tyr or Phe, R₄ is Asp or Asn, R₅ is Trp or Phe, R₆ is Cys, Gly, or Ser, R₇ is Trp or Phe, R₈ is Glu, or Asp, R₉ is Trp or Phe, and wherein at least one of the residues R₁,
50 R₆, R₇, R₈, or R₉ is other than wild-type.

6. An altered pneumolysin as in claim 5 wherein wherein R₁ is Arg, R₂ is Trp, R₃ is Tyr, R₄ is Asn, R₅ is Trp, R₆ is Cys, R₇ is Trp, R₈ is Glu, and R₉ is Trp.
- 5 7. A vaccine including an altered pneumolysin, said altered pneumolysin being non-toxic and being capable of eliciting an immune response in an animal being reactive to wild-type pneumolysin.
8. A vaccine as in claim 7 wherein the altered pneumolysin is as
10 claimed in any one of claims 2 to 6.
9. A vaccine comprising capsular polysaccharide material conjugated with a protein carrier and non-conjugated protein material, the capsular polysaccharide material being derived from any one or
15 more than one of the *Streptococcus pneumoniae* serotypes, and the non-conjugated protein material being an altered pneumolysin, said altered pneumolysin being non-toxic and being capable of eliciting an immune response in an animal reactive to wild type pneumolysin.
- 20 10. A vaccine as in claim 9 wherein the capsular material is derived from any one or more of the *Streptococcus pneumoniae* serotypes 6A, 6B, 14, 18C, 19A, 19F, 23F, 1, 2, 3, 4, 5, 7F, 8, 9N, 9V, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F.
- 25 11. A vaccine as in either claim 9 or 10 wherein the altered pneumolysin is as claimed in as in any one of claims 2 to 6.
12. A vaccine comprising capsular polysaccharide material conjugated with a protein carrier, the capsular polysaccharide material
30 being derived from any one or more than one of the *Streptococcus pneumoniae* serotypes, and the protein carrier being an altered pneumolysin, said altered pneumolysin being non-toxic and being capable of eliciting an immune response in an animal reactive to wild type pneumolysin.
- 35 13. A vaccine as in claim 12 wherein the capsular material is derived from any one or more of the *Streptococcus pneumoniae* serotypes 6A,

6B, 14, 18C, 19A, 19F, 23F, 1, 2, 3, 4, 5, 7F, 8, 9N, 9V, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F.

14. A vaccine as in either claim 12 or 13 wherein the altered
5 pneumolysin is as claimed in any one of claims 2 to 6.

15. A recombinant plasmid including a DNA sequence encoding an
altered pneumolysin as claimed in any one of claims 1 to 6.

10 16. A hybrid host cell including a recombinant plasmid as claimed in
claim 9 said recombinant plasmid including an inducible expression
control operable for expression of said altered pneumolysin encoding
DNA within a host cell.

15 17. A method of producing an altered pneumolysin including the
steps of purifying said altered pneumolysin from an expression system
including a recombinant plasmid with DNA encoding an altered
pneumolysin said pneumolysin being substantially non-toxic and being
capable of eliciting an immune response in an animal reactive to wild
20 type pneumolysin.

18. A method of producing an altered pneumolysin including the
steps of purifying said altered pneumolysin from a culture of a host cell
including a recombinant clone with DNA encoding an altered
25 pneumolysin said pneumolysin being substantially non-toxic and being
capable of eliciting an immune response in an animal said immune
response being reactive to wild type pneumolysin.

19. A method of producing a vaccine including the step of amplifying
30 a recombinant clone encoding an altered pneumolysin, inducing
transcription and translation of said cloned material, the purification of
altered pneumolysin, and the step of conjugating the altered
pneumolysin with a capsular polysaccharide, the altered pneumolysin
having substantially reduced toxic activity as compared with wild type
35 pneumolysin.

20 A method of producing a vaccine as in claim 19 wherein said
altered pneumolysin is as claimed in any one of claims 2 to 6.

21. An altered pneumolysin as hereinbefore described with reference to the examples.

5 22. A vaccine including an altered pneumolysin as hereinbefore described with reference to the examples.

23. A method of producing a vaccine as hereinbefore described with reference to the examples.

10

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K 39/09, 39/40, A61P 31/04 // C07K 14/315		A2	(11) International Publication Number: WO 00/37105
			(43) International Publication Date: 29 June 2000 (29.06.00)
(21) International Application Number: PCT/US99/30390 (22) International Filing Date: 21 December 1999 (21.12.99) (30) Priority Data: 60/113,048 21 December 1998 (21.12.98) US (71) Applicant: MEDIMMUNE, INC. [US/US]; 35 West Watkins Mill Road, Gaithersburg, MD 20878 (US). (72) Inventors: JOHNSON, Leslie, S.; 13545 Ambassador Drive, Germantown, MD 20874 (US). KOENIG, Scott; 10732 Ralston Road, Rockville, MD 20852 (US). ADAMOU, John, E.; 20822 Shamrock Glen Circle, Germantown, MD 20874 (US). (74) Agents: GRANT, Alan, J. et al.; Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein, 6 Becker Farm Road, Roseland, NJ 07068 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>	
(54) Title: STREPTOCOCCUS PNEUMONIAE PROTEINS AND IMMUNOGENIC FRAGMENTS FOR VACCINES			
(57) Abstract			
A vaccine composition is disclosed that comprises polypeptides and fragments of polypeptides containing histidine triad residues or coiled-coil regions, some of which polypeptides or fragments lie between 80 and 680 residues in length. Also disclosed are processes for preventing infection caused by <i>S. pneumoniae</i> comprising administering of vaccine compositions.			

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STREPTOCOCCUS PNEUMONIAE PROTEINS AND IMMUNOGENIC FRAGMENTS FOR VACCINES

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This application is based on U.S. Provisional Application No. 60/113,048, filed 21 December 1998, which is hereby incorporated in its entirety.

10

FIELD OF THE INVENTION

This invention relates generally to the field of bacterial antigens and their use, for example, as immunogenic agents in humans and animals to stimulate an immune response. More specifically, it relates to the vaccination of mammalian species with a polypeptide comprising at least one conserved histidine triad residue (HxxHxH) and at least one helix-forming polypeptide obtained from *Streptococcus pneumoniae* as a mechanism for stimulating production of antibodies that protect the vaccine recipient against infection by a wide range of serotypes of pathogenic *S. pneumoniae*. Further, the invention relates to antibodies against such polypeptides useful in diagnosis and passive immune therapy with respect to diagnosing and treating such pneumococcal infections.

In a particular aspect, the present invention relates to the prevention and treatment of pneumococcal infections such as infections of the middle ear, nasopharynx, lung and bronchial areas, blood, CSF, and the like, that are caused by pneumococcal bacteria.

BACKGROUND OF THE INVENTION

Streptococcus pneumoniae is a gram positive bacteria which is a major causative agent in invasive infections in animals and humans, such as sepsis, meningitis, otitis media and lobar pneumonia (Tuomanen et al. *New Engl. J. Med.* **322**:1280-1284 (1995)). As part of the infective process, pneumococci readily bind to non-inflamed human epithelial cells of the upper and lower respiratory tract by binding to eukaryotic carbohydrates in a lectin-like manner (Cundell et al., *Micro. Path.* **17**:361-374 (1994)). Conversion to invasive pneumococcal infections for bound bacteria may involve the local generation of inflammatory factors which may activate the epithelial cells to change the number and type of receptors on their surface (Cundell et al., *Nature*, **377**:435-438 (1995)). Apparently, one such receptor, platelet activating factor (PAF) is engaged by the pneumococcal bacteria and within a very short period of time (minutes) from the appearance of PAF, pneumococci exhibit strongly enhanced adherence and invasion of tissue. Certain soluble receptor analogs have been shown to prevent the progression of pneumococcal infections (Idanpaan-Heikkila et al., *J. Inf. Dis.*, **176**:704-712 (1997)). A number of various other proteins have been suggested as being involved in the pathogenicity of *S. pneumoniae*. There remains a need for identifying polypeptides having epitopes in common from various strains of *S. pneumoniae* in order to utilize such polypeptides as vaccines to provide protection against a wide variety of *S. pneumoniae*.

SUMMARY OF INVENTION

In accordance with the present invention, there is provided vaccines and

vaccine compositions that include polypeptides obtained from *S. pneumoniae* and/or variants of said polypeptides and/or active fragments of such polypeptides.

- 5 The active fragments, as hereinafter defined, include a histidine triad residue(s) and/or coiled coil regions of such polypeptides.

 The term "percent identity" or "percent identical," when referring to a sequence, means that a sequence is compared to a claimed or described
10 sequence from an alignment of the sequence to be compared (the "Compared Sequence") with the described or claimed sequence (the "Reference Sequence"). The percent identity is determined as follows:

$$\text{Percent Identity} = [1 - (C/R)] 100$$

15

 wherein C is the number of differences between the Reference Sequence and the Compared Sequence over the length of the alignment between the Compared Sequence and the Reference Sequence wherein (i) each base or amino acid in the Reference Sequence that does not have an aligned base or
20 amino acid in the Compared Sequence and (ii) each gap in the Reference Sequence and (iii) each aligned base or amino acid in the Reference Sequence that is different from an aligned base or amino acid in the Compared Sequence, each being a difference; and R is the number of bases or amino acids in the Reference Sequence over the length of the alignment with the Compared
25 Sequence with any gap created in the Reference Sequence also being counted as a base or amino acid.

 If an alignment exists between the Compared Sequence and the Reference Sequence in which the Percent Identity as calculated above is about

equal to or greater than a specified minimum Percent Identity than the Compared Sequence has the specified minimum Percent Identity to the Reference Sequence even though alignments may exist in which the hereinabove calculated Percent Identity is less than the specified Percent Identity.

"Isolated" in the context of the present invention with respect to polypeptides and/or polynucleotides means that the material is removed from its original environment (*e.g.*, the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living organism is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the co-existing materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment. The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

BRIEF DESCRIPTION OF DRAWINGS

Figures 1A-1C, respectively, report the results of three experiments using different preparations of SP36. The results demonstrate that active immunization with recombinant SP36 derived from pneumococcal strain Norway serotype 4 is able to protect mice from death in a model of pneumococcal sepsis using a heterologous strain, SJ2 (serotype 6B). In each of the three experiments shown, one hundred percent of the mice immunized

WHAT IS CLAIMED IS:

1. A vaccine composition comprising:

(a) at least one member selected from the groups consisting
5 of (i) a polypeptide comprising a polypeptide sequence that is at least 90% identical to amino acids 1-819 of SEQ ID NO:4; (ii) a polypeptide comprising a polypeptide sequence that is at least 90% identical to amino acids 1-460 of SEQ ID NO:6; (iii) a fragment of the polypeptide of (i) that includes at least one of a histidine triad residue or coiled-coil region; (iv) a fragment of the
10 polypeptide of (ii) that includes at least one of a histidine triad residue or a coiled-coil region; (v) a fragment of a polypeptide that is at least 90% identical to the polypeptide sequence comprising amino acids 1-800 of SEQ ID NO:8, wherein said fragment includes at least one of a histidine triad residue or coiled-coil region wherein said fragment includes at least 80 amino
15 acids and no more than 680 amino acids; and (vi) a fragment of a polypeptide that is at least 90% identical to the polypeptide sequence comprising amino acids 1-800 of SEQ ID NO:10, wherein said fragment includes at least one of a histidine triad residue or coiled-coil region wherein said fragment includes at least 80 amino acids and no more than 680 amino
20 acids; and

(b) a pharmaceutically acceptable carrier.

2. A process for preventing infection caused by *S. pneumoniae* comprising:

25 administering the vaccine of claim 1.

3. A vaccine composition comprising:

(a) at least one antibody against a member selected from the group consisting of (i) a polypeptide comprising a polypeptide sequence that

is at least 90% identical to amino acids 1-819 of SEQ ID NO:4; (ii) a polypeptide comprising a polypeptide sequence that is at least 90% identical to amino acids 1-460 of SEQ ID NO:6; (iii) a fragment of the polypeptide of (i) that includes at least one of histidine triad residue or coiled-coil region; (iv) 5 a fragment of the polypeptide of (ii) that includes at least one of a histidine triad residue or a coiled-coil region; (v) a fragment of a polypeptide that is at least 90% identical to the polypeptide sequence comprising amino acids 1-800 of SEQ ID NO:8, wherein said fragment includes at least one of a histidine triad residue or coiled-coil region wherein said fragment includes at 10 least 80 amino acids and no more than 680 amino acids and (vi) a fragment of a polypeptide that is at least 90% identical to the polypeptide sequence comprising amino acids 1-800 of SEQ ID NO:10, wherein said fragment includes at least one of a histidine triad residue or coiled-coil region wherein said fragment includes at least 80 amino acids and no more than 680 amino 15 acids.

20

25